FIELD OF THE INVENTION:

This invention relates to a diagnostic kit for detecting pulmonary & extra pulmonary tuberculosis.

BACKGROUND OF THE INVENTION:

The conventional methods for detecting tuberculosis is time consuming & labour consuming AFB staining is considered to be insensitive (requiring 10,000 organism/ml of sputum for smear positive result with 100X microscope, refer Todar's Text Book of Bacteriology Online). ELISA-KP 90 is also known to be of low sensitivity and specificity and specificity (cut-off value >1.0 +ve, and <0.8 –ve test result) and requires sophisticated infrastructure as also the hypersensitivity based Tuberculin Skin Test (Montaux test) which lacks sensitivity and specificity in BCG vaccinated patient (Constantin P.et.al., Inf & Imm 1998;66). In the same way MYCODOT is inconvenient for HIV correlated individuals (refer G.R.Somi et.al., Int J Tubercle and Lung Disease, 19999, vol 3) and Bactec-460 and Roche molecular system PCR based product) are though sensitive requires very costly infrastructure and technical expertise.

OBJECTS OF THE INVENTION:

An object of this invention is to propose a diagnostic kit for detecting tuberculosis.

A further object of this invention is to propose a diagnostic kit which is economical and easy to handle.

A still further object of this invention is to propose a diagnostic kit based on liposome agglutination.

Another object of this invention is to propose a diagnostic kit which is sensitive and specific as it is based on a specific antigenic, antibody reaction.

Still another object of this invention is to propose a diagnostic kit which helps in fast detection of tuberculosis.

BRIEF DESCRIPTION OF THE INVENTION

According to this invention there is provided a diagnostic kit for detecting pulmonary & extra pulmonary tuberculosis comprising a test card "TB Screen" coated with a hydrophobic material, antigen suspension, positive and Negative control.

In accordance to this invention there is provided a method of detecting tuberculosis using the kit comprising

applying positive control, negative control & test sample each in circular motion on the test card coated with hydrophobic material adding said antigen suspension to each of the positive, negative & test sample to interpart the results, clumping of specific antigen and anti body as dark blue agglutination was observed in positive control and the test sample which contain the active tuberculosis infection.

DETAILED DESCRIPTION OF THE INVENTION

The Mycobacteriun tuberculosis $H_{37}Rv$ (ATCC-27294) strains was grown on Saulons media till late log phase (2-3 month) and cells were harvested by centrifugation (5000,-10,000 g for 10-20 min) at 4-10 C, the pellet was washed with PBS (pH 7.2-7.5), resuspended with TEN buffer PH 8.0-8.5 (10mM Tris HC1, 1mM EDTA, 100mM NaCl) and heat inactivated at70-80C (waterbath) for 30-45 min. followed by sonication. The glycolipid antigens were extracted

according to the procedure mentioned in the literature (Reggiardo et al., 1974; Bisen P.S. et al., 2003) with the slightly modification in the procedure. The lypolized Mycobacterial powder (10-15g) was taken into a glass reagent bottle and to it 100-150ml of chloroform and methanol mixture (2:1) was added. This was stirred at room temperature for 50-60 min. and filtered through whatman filter paper No 1. The 1/5 volume of 0.7%KCl (20.0 ml) was added to the filtrate and was shaken for 5-6 times. The suspension was transferred to a separating funnel and kept at 2-8°C for overnight till two distinct layers were separated. The lower organic phase was washed with 1/5 volume of washing solvent (C:M:W:3:48:47) in similar manner by keeping at 2-8°C for overnight. The upper aqueous phase was removed and lower organic phase was retained after filtering. The organic phase was dried by evaporating the solvent in rotatory solvent evaporator at 40-50°C. The moisture was removed by flushing the dried mixture with nitrogen gas. Neutral lipids were removed from the dried mixture by adding 300-500 ml of chilled acetone vortexig it for 10-20 min and filtering it through whatman No.1. This step was repeated till the lipids in the flask became whilish or colorless. This was filtered through whatman No.1 and the filtrate was discarded. The lipids present on the filter paper were dissolved with C:M (2:1) and transferred to the R.B flask. Solvent was rotary evaporated under reduced pressure at 40-50°C. The crude preparation was reconstituted in 10-15 ml of C:M (2:1) and stored at -20°C for further use.

Purification of Antigen(s):

The Silica gel H activated at 100-110°C for 1-1.30 hrs. (Hot air oven) was packed with glass column (2.5X30 cm) with manual tapping and known quantity of crude material (1g/5 ml, stock) was loaded on either side of the column. The column was run ascending on chromatographic jar (4.5X25cm) with purification solvent, 150-200ml (mobile phase) in a ratio of 65:25:4 (C:M: W)[7,8] at room temperature

to run the column till other it reached the end. The column was removed from the chromatographic jar and placed on fume hood to evaporate the solvent from the column. The 1 cm length of each fraction was carefully scrapped using clean rod so as to get the separate molecules which were adsorbed with the silica gel depending upon the mobility and Retardation Factor (RF) value (46.6, 53.4,58.3, 67.2 and 72.4%) of individual molecule. The individual fraction was collected into clean dry glass test tubes which were labeled with respective fraction number. Ten ml of extraction solvent (mixture of chloroform: Methanol 2:1) was added to each test tubes and kept at room temperature for 30-40min. The purity of eluted material was analyzed by TLC and the selected fraction were further filtered through Whatman filter paper No.1 to remove the silica gel from the samples. The pure fractions were pooled and these were characterized by conventional methods (Immuno-staining on TLC, ELISA and by Liposome).

Method for the construction of Liposomal antigens:

Liposome was prepared as described previously (Bangham A.D et. al.1965) with minor modification in the procedure, in brief Phophotidylcholine 100-150mg; cholesterol, 450-500 mg (Sigma, USA); antigenic suspension (Cocktail)10-20 mg; and dye50-100µl (1.0 % sudan black B in Chloroform) were taken in a pre-dried round bottom flask. Solvent was evaporated by rotatory vacuum evaporator under reduced pressure. The dried contents were dissolved in 40-50 ml of absolute alcohol (99.9%Hyman, Germany) and were kept at 4°10°C for 1-1.30 hrs. Sucrose solution (4-8 ml; 150Mm) was taken in a polypropylene centrifuge tube (capacity 35 ml) and to that 4-5 ml of pre-prepared alcoholic antigen suspension was gently added while vortexing. The tubes were kept over night at 4°C-10C for liposome swelling, vortexed with 10-15 ml of PBS (pH 6.5) buffer and centrifuged at 10, 000g for 10-20 min (Beckman, USA). The supernatant was discarded and the pellet was resuspended with 20-30 ml of B2 buffer, pH 7.2 ($NaH_2PO_4.2H_20$, 10mM; KH_2PO_4, 10mM; EDTA, 10mM; Choline Chloride, 10% and Thiomersol, 0.1%). This was stored at 4°C-10C for further use and utilized as antigen reagent for the kit.

Preparation of Phosphatidylcholine:

Phosphatidylcholine (PC) was prepared in house to reduce the cost of the test. Those skilled in the art are aware of purification of PC from egg yolk and it's final estimation (Sunamoto, J.et al 1978). In brief, 25 eggs were taken and albumin portions were removed. The collected yolk was extracted with 750 ml of Chloroform: Methanol (2:1) by stirring for half an hour. Filtered through Whatmann no. 1. The filterate was deproteinised with one fifth volume of 0.7% of KC1. To the organic layer so obtained, washing was performed with 3:48:47 of Chloroform: methanol: water. Moisture was removed by using benzene. Solvent was evaporated with the aid of rotary vacuum evaporated with the aid of rotary vacuum evaporator and a dried film of lipid was obtained. Neutral lipids were removed as described above with acetone. Empty weight of round bottom flask was taken (Wa). Flask was weighed along with the dried lipid film. 37.5 g of crude lipid was isolated. The crude product was further purified by silica gel H chromatography and purified PC was characterized by Thin layer chromatography and PC estimation was performed as known in the prior art (Sunamoto, J.et al 1978).

Preparation of positive control for test:

1 mg of Mycobacterium tuberculosis pellet was taken after centrifugation of mycobacterial growth in Sauton's medium. The pellet was washed twice with 1X PBS to get rid of media remnants. The pellet was then suspended in 4 ml of 1X Phosphate buffered saline. 4-8 acid washed beads of 5 mm diameter was added to the above. The sample was vigorously shaken for 10 mm on a vortex. The suspension obtained was mixed with an equal volume of Freunds Incomplete Adjuvant. The mixture was squeezed through 22g needle repeatedly till it reaches a desired level. 100 ul of suspension was inoculated to a young rabbit of 2-8 months. A number of rabbits were inoculated in the same manner.

Rabbits were bled after one month and serum was obtained. The reactivity of serum was checked with the antigen suspension as described in the test procedure given in next paragraph. The reactivity titer was checked. A booster administration of the antigen (50 ul) was again repeated. An enhanced titer of about 1:64 to 1:128 was obtained after about seven days of booster dosage. Best reactivity titer was obtained. The serum was diluted to optimum reactivity titer in 1X PBS and 0.1% azide was added as preservative so as to contain any contamination. The stock was frozen till used/dispensed in vials.

The 4-6 month old Rabbits were immunized with the above antigens and bled periodically and used for positive control to be provided with the kits, where as normal young Rabbit were used for Negative control.

Method of testing:

All the components such as positive control, negative control, antigen suspension and sample to be tested were brought to room temperature before performing the experiments. Positive control, negative control and test sample (25µl each) were added in circular motion, as demonstrated on the test card. The above samples were spread by using separate sticks in round conjugation, 25 µl of liposome antigen was added to it and the card was gently swirled for 4 min.

Freshly procured or frozen test serum samples (25 µl) were spread evenly inside the circular zone of hydrophobic material coated plastic slide. For convenience, zone 1 and 2 were spread with the positive (anti-rabbit serum) and negative control (normal rabbit serum) and negative control (normal rabbit serum) respectively, to interpret the results. The antigenic suspension (25 µl) was added to each circular zone including zone 1 and 2 and the card was manually swirled for 4 min. The clumping of specific antigen and antibody as dark blue agglutination were observed in positive control as well as in those samples which contain antibodies against mycobacterial glycolipid with active tuberculosis

infection. No clumping on the card whereas, indicated a negative result. The peripheral drying on the circular zone indicated indiscriminate results, which require further confirmation within 15-30 days, as these samples contained undetectable level of antigen concentration in the specimens.

EXAMPLE 1

SERA:-

Patient sera from outdoor patient departments (OPD) from different hospitals of India were enrolled in the present study to cover maximum population diversity. The patients were diagnosed on the basis of clinical and radiological evaluation as well as smear staining and sputum culture of samples. None of the patients was completely treated. Both extrapulmonary as well as pulmonary tuberculosis sera were included in the study. A total of three hundred and twenty four (324) tuberculosis sera were studied.

Sera from healthy individuals without any clinical symptoms of TB were included as negative controls to evaluate specificity criterion of the test. Most of these were obtained from BCG vaccinated subjects. The non-TB sera generally belonged either to healthy individuals or to patients suffering from a variety of diseases other than tuberculosis. The sera were stored frozen and were used within 1 year from the time, they were taken. Also five hundred and eleven (511) tuberculosis negative sera were included in this testing. The details of criteria used in selection of sera is as follows:

- Smear Negative, Culture Positive pulmonary cases ---52
- Smear Positive, Culture Positive, pulmonary cases ---180
- Extrapulmonary, Culture Positive cases ---35
- Relapse pulmonary cases ---57
- Drug treated, clinically negative cases ---60
- Healthy household contacts ---50

- BCG vaccinated children ---15
- Hepatitis B positive samples ---15
- Sera from common infections other than TB ---27
- Normal human sera ---344

EXAMPLE 2

IN HOUSE EVALUATIONS:-

An overall sensitivity of 98.68% was obtained using a panoply of three hundred and twenty four tuberculosis sera, out of which 20 sera showed indiscriminate results. Indiscriminate sera were not included in the sensitivity and specificity calculations, as per method adopted by WHO. An overall specificity of 98.78% was obtained using five hundred and eleven non-tuberculosis sera.

Sera from 15 children who were recently immunized with BCG were tested for any cross-reactivity of the test with vaccination. None of the sera yielded positive results, thereby indicating the suitability of the test in BCG vaccinated populations such as India and others.

15 cases of Hepatitis B positive samples were evaluated for cross reactivity. There was no reaction in any of the sera tested. 4 Hepatitis B sera were tested with the kit at Hopkins Research Institute, Bombay with nil reactivity (not included in inhouse study table).

Out of 27 sera from other common infections, 25 showed clear negative and rest two showed indiscriminate results. There was a need to chase these subjects for progression to tuberculosis, but unfortunately, it could not be done. Indeterminate results were omitted from specificity and sensitivity calculations.

Results of inhouse studies are tabulated as follows:

Table 1

STUDIES PERFORMED ON TUBERCULOSIS POPULATION

SERA DETAILS	RESULTS
• Number of tuberculosis sera tested=324	positive=300
	negative=4
	indeterminate=20**
	SENSITIVITY= 98.68%
Smear Negative, Culture Positive pulmonary cases ---52	positive=47 negative= 2 indeterminate=3
Smear Positive, Culture Positive, pulmonary cases --- ---180	positive = 175 negative = --- indeterminate =5
Extrapulmonary cases, Culture Positive ---35	positive=28 negative =2 indeterminate=5
Relapse pulmonary cases ---57	positive=50 negative= --- indeterminate = 7

**indiscriminate samples were not included in Sensitivity & specificity calculations as per WHO methodology

Table 2

STUDIES PERFORMED ON NON-TUBERCULOUS SERA

• Number of non-TB sera tested =511	positive =488
	negative=6
	indeterminate=17**
	SPECIFICITY=98.78%
5. Drug treated, clinically negative cases	positive= ---
---60	negative=51
	indeterminate=9
6. Healthy household contacts ---50	positive= ---
	negative=50
	indeterminate= ---
7. BCG vaccinated children ---15	positive= ---
	negative=15
	indeterminate= --
8. Hepatitis B positive samples ---15	positive= ---
	negative= 15---
	indeterminate =
9. Sera from common infections (Not TB)	positive = --
--- 27	negative=25
	indeterminate = 2
10. Normal human sera ---344	positive = 6
	negative = 332
	indeterminate = 6

**indiscriminate samples were not included in Sensitivity & specificity calculations as per WHO methodology

Excellent results were obtained when using fresh sera from subjects under investigation. Frozen sera can be tested after thawing, but repeated freeze thawing of samples might affect the outcome.